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Simultaneous detection of endosulfan and chlorpyrifos residues in buffalo meat using HPLC

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ABSTRACT

In the present study, the methodologies for extraction, cleanup, detection and quantification of endosulfan α, endosulfan β, endosulfan sulfate and chlorpyrifos residues in buffalo meat were standardized. For the quantification of pesticide residues in the fortified meat tissue samples, first a standard calibration curve was obtained by running different dilutions of standard pesticides. The buffalo meat samples fortified with different known concentrations of endosulfan and chlorpyrifos standards were subjected to extraction with acetonitrile, followed by homogenization, sonication, centrifugation and filtration. The extracts were then subjected to liquid-liquid partition. The extracts were cleaned up by performing alumina column chromatography. HPLC was performed by using isocratic mobile phase consisting of acetonitrile:water(67:33) with the flow rate of 1ml/min and run time of 18 min. the detection wavelength was set at 202nm with 360 nm as the reference wavelength. On HPLC analysis, the limit of detection was recorded to be 0.039μ/g and 0.02929μg/g for endosulfan(α, β and sulfate) and chlorpyrifos, respectively. The recovery percentage obtained was 83.11±2.65, 96.70±3.00, 84.47±3.44 and 86.17±4.09 for endosulfan α, endosulfan β, endosulfan sulfate and chlorpyrifos, respectively.

KEYWORDS

Buffalo meat;
Endosulfan;
Chlorpyrifos;
Limit of detection;
Recovery percentage.

INTRODUCTION

Buffalo meat is known for its high nutritional quality as it is low in calories(143 kcal/100g), cholesterol(82mg/100g) and fat(2.42g/100g), and is rich in iron(3.42 mg/100g), vitamin B_{12}, selenium, zinc, phosphorus, vitamin B_6 and niacin (http://www.eatorganicbuffalo.com). India produces about 1.483 million metric tons (MT) of buffalo meat which is roughly 25% of the total meat production(6.03 million MT)[1]. Besides contributing for indigenous consumption, buffalo meat contributes to about 70%(0.344 million MT) of the total meat ex-

ported from India[2]. Inspite of all these facts, in India the research on pesticide residues in meat in general and buffalo meat in particular has been mediocre.

As per the registration committee under the insecticides act, (1968) India, as many as 181 pesticides have been registered for regular use in the country[3]. And presently, 44 types of pesticides are manufactured in India and endosulfan (6,7,8,9,10,10-hexachloro-1,5,5a,6,9, 9a-hexahydro-6,9-methano-2,4,3-benzadioxathiepin 3-oxide) and chlorpyrifos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phophorothioate) are in common use[4]. Though the consumption of pesticides in India is 0.5kg/ha which is far less vis-α-vis other countries(7kg/ha in the USA) [3], the inadequate knowledge of farmers has led to indiscriminate and injudicious use of pesticides causing the problem of pesticide residues in food.

Endosulfan is an organochlorine pesticide of the cyclodiene subgroup. Technical grade endosulfan is comprised of two molecular forms (isomers), the alpha- and beta-isomers in the ratio of 7:3, respectively[5]. Endosulfan sulfate is a biotransformation reaction product of technical-grade endosulfan[6]. Endosulfan residues in food may affect central nervous system, kidney, liver and blood chemistry. Endosulfan causes teratogenic effects[7]; the use of endosulfan in plantation resulted in large number of children born with deformed limbs and mental abnormalities in Padre village in Kerala state[8]. It also shows mutagenic and carcinogenic effects[9,10]. It is a persistent organic pollutant (POP), for it persists in the environment for extended periods of time(an estimated half-life of 9 months to 6 years) and hence has a high potential for bioaccumulation and biomagni fication[11], increasing the exposure risk of many non-target animals and human beings.

Chlorpyrifos(O,O-diethyl O-3,5,6-trichloro-2-pyridyl phophorothioate) is a non polar, broad-spectrum organophosphorous non-systemic insecticide. It primarily affects the nervous system through inhibition of cholinesterase, an enzyme required for proper nerve functioning. It also affects the cardiovascular and respiratory systems and reports exist on the effects of chlorpyrifos on reproductive and endocrine system[12].

In the present study efforts were made to standardize suitable methodologies for extraction, cleanup, detection and quantification of endosulfan and chlorpyrifos residues in buffalo meat by using high performance liquid chromatography.

EXPERIMENTAL

Chemicals

HPLC grade solvents such as acetonitrile(MeCN), water and dichloromethane, and GR grade chemicals such as alumina(aluminium oxide, neutral activity I-II grade), sodium sulfate were used. Pure technical grade endosulfan α(97 % purity), endosulfan β(99 %), endosulfan sulfate(98 %)(Supelco, USA) and chlorpyrifos (98.5 %)(Sigma-Aldrich Co.) were used.

Apparatus

A homogenizer(Polytron®), a refrigerated centrifuge (Multifuge 1 S-R®), an ultrasonicator(Soniprep®) and a vacuum manifold pump were used.

HPLC system

Perkin Elmer®(model series 200) comprising of quaternary LC pump, autosampler with Rheodyne injector having a 200µl loop, diode array detector and peltier column oven was used.

Column

LichroCART® 250-4 / LiChrospher® 100 RP-18e endcapped(250mm×4mm, with the particle size of 5µm) was used.

Mobile phase

The mobile phase consisted of acetonitrile and water in the ratio of 67:33. The flow rate was 1ml/min and the run time was 18min.

Meat samples

Meat tissues were collected from freshly slaughtered buffaloes at Bareilly slaughter house.

Extraction and cleanup

The buffalo meat samples detected negative for endosulfan and chlorpyrifos residues were fortified with different known concentrations of standard endosulfan and chlorpyrifos. The extraction and cleanup of the residues of endosulfan and chlorpyrifos were carried out as per the method of Bottomley and Baker(1984)[13] with suitable modifications.

The fortified meat tissue sample weighing 5g was

taken in a clean and dry spouted beaker(50ml capacity) and cut into small pieces (less than 1mm thickness) using a clean scissors. Due care was taken to avoid unnecessary contamination of the sample during processing. The sample(weighing 5g) was added with 30ml of acetonitrile and stirred. The stirred mixture was homogenized using high speed homogenizer at 1000rpm for 5min. The homogenate was then sonicated at 15 microns amplitude for 30 cycles with a stop time of 5 seconds at low temperature, which was maintained with crushed ice. The sonicated homogenate was centrifuged at 10,000rpm(4^0C) for 10min and the supernatant was taken and filtered through Whatman (# 42) filter paper. The sediment that remained on the filter paper along with the filter paper was once again homogenized and filtered. Both the filtrates were pooled in a 250ml capacity separatory funnel and added with 50 ml of sodium sulfate solution(2.5%) and 30ml of dichloro methane and then subjected to liquid-liquid partition. The contents were vigorously shaken for 2-5min and kept undisturbed for 10min for the separation of layers. Once the layers got separated, the lower organic layer was collected into a clean and dry beaker. The upper aqueous layer was again subjected to liquid-liquid partitioning with 20ml of dichloromethane and the lower organic layer was collected. The pooled lower organic layer was then dehydrated on sodium sulfate column(a clean test tube of 1cm internal diameter was punched at the bottom and non absorbent cotton was placed at the bottom and then the column was prepared by putting anhydrous sodium sulfate into the tube for the approximate column length of 5cm). The dehydrated extract was allowed to evaporate at room temperature to get approximately 5ml of final volume. This was followed by cleanup by alumina column chromatography.

The content(5ml) obtained in the extraction procedure was subjected to adsorption chromatography on alumina column in order to eliminate various co-extractives such as fats and fatty acids which could possibly be present in the extract and hinder in the detection of peaks of the putative pesticides. The alumina columns were prepared by slurry packing 10g aluminium oxide and dichloromethane(20ml) in the burettes. All possible care was taken to avoid trapping of air bubbles in the column. The five milliliter extract containing the putative pesticide residues was passed through the alumina column. The residues retained were eluted with 10 ml of dichloromethane. The eluate was collected in a beaker and was allowed to evaporate completely under the gentle stream of air.

Detection of endosulfan and chlorpyrifos residues by HPLC

The content of the beaker was reconstituted in 1ml acetonitrile and filtered through 0.22μ millipore membrane filter. A volume of 20μl of this reconstituent was injected into the column for HPLC run. Chromatography was performed by using diode array detector (DAD) at 202nm detection wavelength with 360nm as the reference wavelength. The temperature was kept constant at 40^0C. The run and analysis of samples was carried out by using 'Total Chrom' software.

Quantification of endosulfan chlorpyrifos residues

For the quantification of endosulfan and chlorpyrifos residues in the fortified meat tissue samples, first a standard calibration graph was obtained by running different dilutions of standard endosulfan and chlorpyrifos. Stock standard solution of 100μg ml^{-1} concentration was prepared by dissolving 10mg each of standard endosulfan α, endosulfan β, endosulfan sulfate and chlorpyrifos separately in 100ml each of acetonitrile. From endosulfan stock solution, 200μl was diluted with 800μl of acetonitrile so as to get a working standard solution of 20μg ml^{-1} concentration. With this working standard solution, two-fold dilutions, viz., 20.00, 10.00, 5.00, 2.50, 1.25, 0.625, 0.312, 0.156, 0.078, and 0.039μg ml^{-1} were prepared. From chlorpyrifos stock solution, a volume of 150μl was diluted with 850μl acetonitrile so as to get working standard solution of 15μg ml^{-1} concentration. This working standard solution was further diluted to get two-fold dilutions, viz., 15.00, 7.50, 3.75, 1.875, 0.9375, 0.46875, 0.234375, 0.117187, 0.05859 and 0.029295μg ml^{-1}. An aliquot of 20μl of these concentrations (thrice) was injected into the column for HPLC run and a standard curve was obtained by plotting concentration versus the peak area (average).

Recovery analysis

The area of peaks of standard curves and curves of fortified samples corresponding to the similar concentration were compared and recovery percentage was

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calculated by using the following formula[14]. The recovery percentage obtained was used for the estimation of correction factor. This correction factor could be used to calculate the actual concentration of pesticide residues in the test samples. The formulae used are;

$$\text{Recovery}(\%) = \frac{N(\Sigma xy) - (\Sigma x)(\Sigma y)}{N(\Sigma x^2) - (\Sigma x)^2} \times 100$$

N = number of observations, x = amount of standard pesticide, y = amount of pesticide detected in the fortified samples

$$\text{Correction factor}(C.f.) = \frac{100}{\text{Percent recovery}} \times 100$$

RESULTS AND DISCUSSION

Extraction and cleanup of endosulfan and chlorpyrifos residues

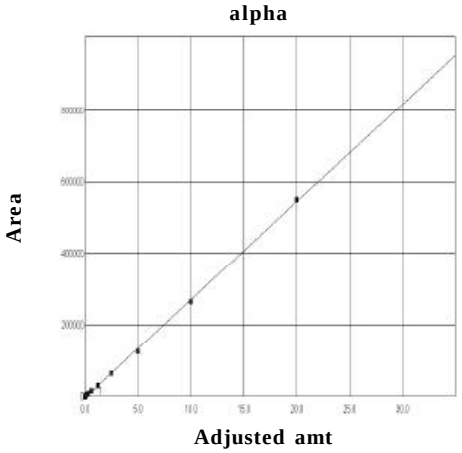
In the current study, the tissue samples were subjected to extraction and cleanup as per the method of Bottomley and Baker[13] with suitable modifications. The solvent acetonitrile was used for the extraction of endosulfan and chlorpyrifos residues from the fortified meat tissue samples, which yielded good recovery percentage (83-97%). Several workers have also reported the use of acetonitrile for the extraction of endosulfan and chlorpyrifos residues from the food of animal origin with good recoveries[13,14,15]. The use of acetonitrile for the extraction of chlorpyrifos from the food of animal origin was also reported by Claborn and Chau[19]; Félix et al.[12] and Atillo et al.[13]. The extraction of endosulfan and chlorpyrifos residues was followed by liquid-liquid partitioning with sodium sulfate solution(2.5%): dichloromethane(1:1 v/v). However, liquid-liquid partition with acetonitrile:hexane was used to extract endosulfan[14,15], organochlorines (including endosulfan)[16] and chlorpyrifos[12] from the food of animal origin. During the course of the present study, it was observed that the combination of sodium sulfate and dichloromethane used for liquid-liquid partition, required lesser evaporation time vis-a-vis acetonitrile:hexane combination. Although Singh and Chawla[17] extracted organochlorines from animal tissue matrices by using liquid-liquid partitioning with hexane:acetone(2:1v/v), the present study revealed the presence of endosulfan in both the organic and aqueous phases. The extract obtained after liquid-liquid partition was dehydrated on the sodium sulfate column to get rid of its water content.

In order to eliminate the co-extractives, adsorption chromatography on alumina column(neutral grade) was performed. Similar(Alumina column) chromatography procedures have also been used for the cleanup of endosulfan and chlorpyrifos residues from the food of animal origin by several researchers[18,19]with recovery of the residues ranging from 75-90%.

Detection and quantification of endosulfan and chlorpyrifos residues

In this study, HPLC with diode array detector (DAD) was used for the detection and quantification of endosulfan and chlorpyrifos residues. High performance liquid chromatography has been used for the quantification of chlorpyrifos[9,20]. The sensitivity of the technique is adjudged on the basis of limit of detection of a particular pesticide residue. In the present study, the limit of detection for endosulfan(α, β and sulfate) was recorded to be 0.039μg/g (Figures 1-3), which was well below the codex maximum residue limit(MRL) of 0.1μg/g for endosulfan in buffalo meat. The limit of de-

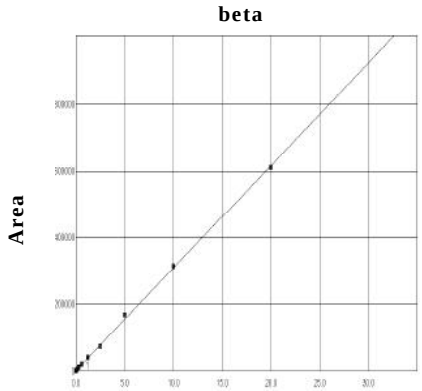
S.no.	Concentration (ppm)	Area
1	0.03905	1589.4
2	0.07810	3652.74
3	0.15620	5987.25
4	0.31250	10425.79
5	0.62500	17214.78
6	1.25000	32478.54
7	2.50000	65924.46
8	5.00000	124789.89
9	10.00000	264578.21
10	20.00000	547895.32



R-Squared=0.999289, Y = (-1144.882838) + (27176.460259) X

Figure 1 : Calibration graph of standard endosulfan α

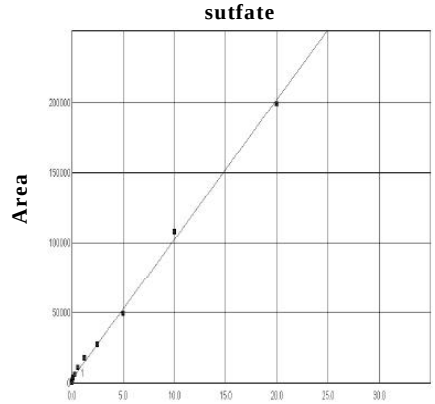
S.no.	Concentration (ppm)	Area
1	0.03905	3012.79
2	0.07810	5247.86
3	0.15620	7854.12
4	0.31250	13245.24
5	0.62500	22897.45
6	1.25000	42150.14
7	2.50000	74879.52
8	5.00000	168754.25
9	10.00000	312546.88
10	20.00000	609746.51



R-Squared = 0.999381, Y = (4234.491550) + (30483.036910) X

Figure 2: Calibration graph of standard endosulfan β

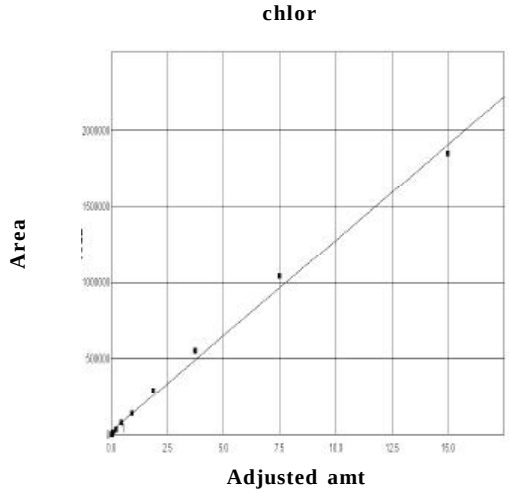
S.no.	Concentration (ppm)	Area
1	0.03905	1198.25
2	0.07810	2548.21
3	0.15620	4125.85
4	0.31250	6744.82
5	0.62500	10854.48
6	1.25000	18457.24
7	2.50000	27800.79
8	5.00000	49875.46
9	10.00000	108245.32
10	20.00000	198542.24



R-Squared = 0.998080, Y=(3342.215186)+(9884.022631) X

Figure 3: Calibration graph of standard endosulfan sulphate

S.no.	Concentration (ppm)	Area
1	0.02929	5421.12
2	0.05859	13385.15
3	0.11718	21141.89
4	0.23437	40015.87
5	0.46875	82547.02
6	0.93750	139975.46
7	1.87500	294571.19
8	3.75000	554712.01
9	7.50000	1041206.79
10	15.00000	1845245.20



R-Squared= 0.995307, Y=(30212.523613)+(124658.314170) X

Figure 4: Calibration graph of standard chlorpyrifos

tection for chlorpyrifos was recorded to be 0.02929µg/g (Figure 4), which was well below the MRL of chlorpyrifos (1µg/g [CODEX] and 0.1µg/g [Bureau of Indian Standards]) which accentuates the validity of the method. The calibration graphs for endosulfan α, endosulfan β and endosulfan sulfate (Figures 1-3) were found to be linear from 0.03905 to 20µg/ml with a correlation coefficient of 0.9992, 0.9993 and 0.9980 respectively. The calibration graph for chlorpyrifos (Figure 4) was also found to be linear from 0.02929 to 15µg/ml with a correlation coefficient of 0.9953. These graphs were used to calculate the concentration of endosulfan and chlorpyrifos residues in the fortified samples and could be used for test samples.

In order to get higher accuracy in the detection of pesticide residues, selection of suitable wavelength is very important. During the course of collection of spectra in this study, all the four pesticides(endosulfan α, endosulfan β, endosulfan sulfate and chlorpyrifos) found to absorb light at the wavelength of 198-207nm. Thus, the wavelength of 202nm(average) was selected in the

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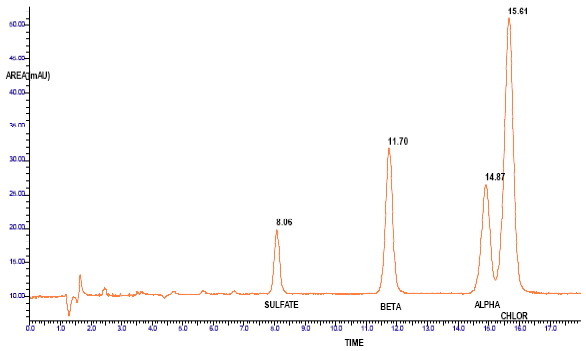

Figure 5 : HPLC chromatogram of standard endosulfan α, endosulfan β, endosulfan sulfate (20ppm each) and chlorpyrifos (15ppm) together

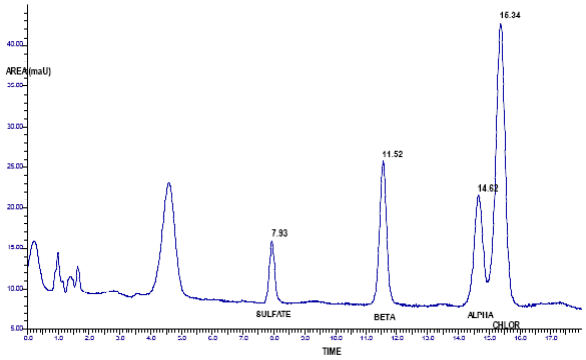

Figure 6 : HPLC chromatogram of sample spiked with endosulfan α, endosulfan β, endosulfan sulfate (20ppm each) and chlorpyrifos (15ppm) together

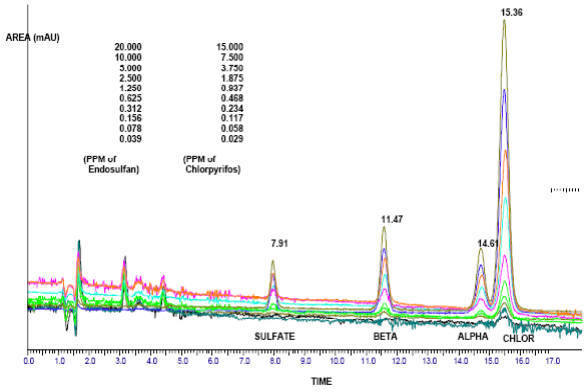

Figure 7 : HPLC chromatograms (superimposed) of different dilutions (ppm) of standard endosulfan α, endosulfan β, endosulfan sulfate and chlorpyrifos

present study with a reference wavelength of 360 nm. It yielded stable baseline, less interfering peaks and good amplitude of the chromatograms. This wavelength (202nm) has also been employed by Iorger and Smith[9]. In other studies, Reuke and Hauck[21] employed a wavelength of 220 nm for the detection of endosulfan residues. But in the present study, when the standards were run in the dual mode(202 and 220nm), the sensitivity recorded was higher at 202 nm compared to 220nm.

The isocratic mobile phase of acetonitrile:water (67:33) was used to perform the high performance liquid chromatography as it showed least background interference because of low absorbance of acetonitrile in 198 to 220nm range of wavelength. This result is in accordance with the revelations of Moye[22]. Several workers have also used acetonitrile:water as the mobile phase for the detection of endosulfan[21] and for chlorpyrifos[23] but at slightly different ratio of 65:35. At the ratio of 67:33, it was observed that all the four pesticides (endosulfan α, endosulfan β, endosulfan sulfate and chlorpyrifos) got eluted earlier than the one that eluted at 65:35 with the same amplitude and separation of the peaks (Figures 5 and 6) giving advantage of reducing the run time. Using this mobile phase, the pesticides, *viz.*, endosulfan α, endosulfan β, endosulfan sulfate and chlorpyrifos on an average got eluted at 14.6(14.32-14.88), 11.52(11.25-11.82), 7.94(7.69-8.10) and 15.35(15.25-15.85) minutes(TABLE 1), respectively.

To estimate the recovery percentage of endosulfan and chlorpyrifos residues, the samples detected negative for these pesticide residues were fortified with different known concentrations of standard endosulfan and chlorpyrifos. On HPLC analysis, the recovery percentage (± S.E.) obtained were 83.11±2.65, 96.70±3.00, 84.47±3.44 and 86.17± 4.09 for endosulfan α, en-

TABLE 1 : Retention time, recovery percentage and correction factor for endosulfan and chlorpyrifos in fortified buffalo meat samples

Pesticide	Mean retention time(Min.) (range)	Mean recovery % (± S.E.)	Correction factor
Endosulfan α	14.6 (14.32-14.88)	83.11± 2.65	1.215
Endosulfan β	11.52 (11.25-11.82)	96.70 ± 3.00	1.034
Endosulfan sulfate	7.94 (7.69-8.10)	84.48 ± 3.44	1.183
Chlorpyrifos	15.35 (15.25-15.85)	86.17 ± 4.09	1.160

S.E.: Standard Error

dosulfan β, endosulfan sulfate and chlorpyrifos, respectively (TABLE 1 and Figures 5 and 6). In all the cases, more than 80% recovery of residues was obtained which indicated the suitability of the methodologies developed in this study. The correction factors calculated by using the values of recovery percentage are 1.215, 1.034, 1.183 and 1.160 for endosulfan α, endosulfan β, endosulfan sulfate and chlorpyrifos, respectively.

CONCLUSION

Based on the results of this study, 18 minute run time, good limit of detection (sensitivity) and recovery percentage, the methodologies developed could be used to detect endosulfan and chlorpyrifos residues in field samples. The correction factors could be used to quantify endosulfan and chlorpyrifos residues in field samples. This method could be applied and is open for further research to detect residues of other pesticides.

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